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Role of Cationic Residues in Cytolytic Activity: Modification of Lysine Residues in the Cardiotoxin from *Naja nigricollis* Venom and Correlation between Cytolytic and Antiplatelet Activity†

R. Manjunatha Kini and Herbert J. Evans*
Department of Biochemistry and Molecular Biophysics, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

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ABSTRACT: Cardiotoxins and postsynaptic neurotoxins from snake venoms have similar primary, secondary, and tertiary structures. Cardiotoxins, however, in contrast to neurotoxins, exhibit general cytotoxicity. Comparison of the distribution of hydrophobic and charged amino acid residues in the three-dimensional structures of lytic cardiotoxins and nonlytic neurotoxins indicates the presence of a cationic site associated with a hydrophobic surface in cardiotoxins, but not in neurotoxins. A cationic site flanked by a hydrophobic site is a common structural feature shared by a wide variety of unrelated cytolysins and is predicted to determine the lytic activity of a large group of cytolysins. To determine the essential nature of the cationic site in cardiotoxin CTX-1 from *Naja nigricollis crawshawii* venom, we modified the positive charges of nine Lys residues to negative, neutral, or positive charges by succinylation, carbamylation, or guanidination, respectively. Circular dichroism studies indicated that these modifications did not affect the conformation of the cardiotoxin. Binding of the modified cardiotoxins to phospholipids was demonstrated by changes in the intrinsic fluorescence of native and modified CTX-1 after binding to phospholipid vesicles, and by resonance energy transfer with anthracene–phospholipid vesicles. Phospholipid binding was not affected by these modifications, but their binding preference was determined by the electrostatic interactions between the polypeptide and phospholipid. Both positively charged native and guanidinated CTX-1 showed direct lytic activity on human erythrocytes and platelets, whereas the succinylated or carbamylated derivatives did not show lytic activity. The loss of lytic activity cannot be related to conformational changes or phospholipid binding abilities of the modified cardiotoxins. Thus, these results suggest a significant role of the positive side chains of Lys residues in determining the lytic activity of CTX-1. We demonstrated earlier that the antiplatelet effects shown by the cardiotoxins are due to their lytic ability. This is further supported by the observed antiplatelet effects of lytic native or guanidinated CTX-1. Nonlytic succinylated or carbamylated CTX-1 failed to affect platelet aggregation, supporting the proposed lytic mechanism for antiplatelet effects.

Snake venoms are complex mixtures of toxic proteins and polypeptides. Among these toxins are neurotoxins and cardiotoxins, the family of polypeptides of molecular weights 6000–7500, which have been well characterized (Dufton & Hider, 1983, 1988). Neurotoxins, which block neurotransmission postsynaptically at the nicotinic site of acetylcholine receptors, belong to 2 classes: short-chain neurotoxins with 60–64 amino acid residues and 4 disulfide bridges and long-chain neurotoxins with 65–74 amino acid residues and 5 disulfide bridges. These two groups have high structural homology and share common postsynaptic activity (Dufton & Hider, 1983, 1988). Cardiotoxins, which are isolated only from elapid snake venoms, closely resemble short-chain neurotoxins, with 60 amino acid residues and 4 disulfides. Despite the structural homology, cardiotoxins are more basic and more hydrophobic than the neurotoxins, and differ from neurotoxins in their pharmacological properties. Cardiotoxins exhibit depolarization of cardiac, skeletal, and smooth muscles resulting in muscle contracture, depolarization of nerve cells leading to loss of excitability, general cytolysis, and a weak anticoagulant effect (Dufton & Hider, 1983, 1988; Harvey, 1985; Condrea, 1974; Teng et al., 1987; Kini et al., 1987).

We purified four nonenzymatic, anticoagulant, and antiplatelet polypeptides from *Naja nigricollis crawshawii* venom (Kini et al., 1987) and identified these polypeptides as cardiotoxins on the basis of their amino acid composition, amino-terminal sequences, and direct lytic activity on human erythrocytes (Kini et al., 1988). The cardiotoxins inhibit aggregation of platelets, and we demonstrated that the observed inhibition is merely a reflection of the lytic ability of these cardiotoxins (Kini & Evans, 1988). Lysis of blood cells and release of inhibitory substances, and lysis of platelets coated on electrodes, lower the impedance, and thus result in apparent inhibition of aggregation on a whole blood aggregometer. In support of this, we also showed that neurotoxin II from *N. naja oxiana* venom fails to exhibit any platelet

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* To whom correspondence should be addressed.

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effect (Kini & Evans, 1988). Because of its structural homology, but lack of lytic activity, neurotoxin II provided a good negative control. To further establish the role of cytolytic activity in determining the platelet effects of the cardiotoxins, we have now prepared nonlytic cardiotoxin derivatives by specific chemical modification of amino acid residues.

Recently, we identified a common cytolytic region in a wide variety of cytolysins isolated from microbial, insect, amphibian, reptilian, and mammalian origins (Kini & Evans, 1989). Irrespective of their offensive or defensive roles, or their cellular targets, these cytolysins all possess a characteristic cationic site flanked by a hydrophobic surface. In this paper, we examine the role of the cationic residues in the cytolytic activity of cardiotoxins. The Lys residues of cardiotoxin 1 (CTX-1)[1] of *N. nigricollis crawshawii* venom were succinylated, carbamylated, or guanidinated to see the effects of these modifications on erythrocyte and platelet lysis, and on platelet aggregation. The results provide evidence for the essentiality of the cationic residues for cytolytic activity, and give additional proof that the antiplatelet effects of cardiotoxins are linked to cell lysis.

Materials and Methods

Venom and Reagents. *Naja nigricollis crawshawii* venom, PC from hen's egg, cardiolipin, dansyl chloride, *O*-methylisourea, and hydroxylamine were purchased from Sigma Chemical Co., St. Louis, MO. 4-Vinylpyridine and succinic anhydride were obtained from Aldrich Chemical Co., Milwaukee, WI, and potassium cyanide was from J. T. Baker Chemical Co., Phillipsburg, NJ. Normal human blood for erythrocytes and for platelet aggregation studies was freshly drawn from healthy volunteers. All other reagents were analytical grade.

Purification and Homogeneity of Cardiotoxin CTX-1. The major cardiotoxin, CTX-1, from *N. nigricollis crawshawii* venom was purified by fast protein liquid chromatography on a Pharmacia Mono S column followed by gel filtration on a Bio-Gel P-10 column (Kini et al., 1987). CTX-1 was rechromatographed 3 times on Mono S and 2 times on a gel filtration column to minimize phospholipase contamination. The cardiotoxin was found to be homogeneous on both native and sodium dodecyl sulfate–polyacrylamide gels, and phospholipid-splitting activity was undetectable (<0.002% contamination with phospholipase) by a standard pH stat assay, using an egg suspension without detergents as substrate (Nieuwenhuizen et al., 1974).

Reduction and Pyridylethylation. The cardiotoxin was reduced and pyridylethylated according to the method of Cavins and Friedman (1970). Protein (10 mg/mL) in 6 M guanidine hydrochloride/0.13 M Tris, pH 8.0, was treated with β-mercaptoethanol (20 mol/mol of disulfide). After incubation at room temperature for 3 h, the mixture was treated with 3 mol of 4-vinylpyridine per mole of β-mercaptoethanol. After 90-min further incubation, the mixture was desalted on a Sephadex G-25 column equilibrated with 9% formic acid. This pyridylethylated CTX-1 was used only in the sequence determination.

Amino Acid Analyses and Sequence Determination. Amino acid analyses and sequence determinations were carried out as described earlier (Kini et al., 1988). Sequence analysis was performed on pyridylethylated CTX-1 in an Applied Biosystems 470A protein sequencer with an Applied Biosystems 120A PTH analyzer.

Modification of Free Amino Groups. Free amino groups of CTX-1 were modified by three independent methods.

(*A*) *Succinylation.* Native CTX-1 was succinylated according to the method of Klotz (1967). One hundred moles of succinic anhydride per mole of amino groups (nine ϵ-amino groups of Lys and the free α-amino group) was added in small aliquots to a rapidly stirred solution of CTX-1. The pH was maintained between 8.0 and 8.5 by addition of 1 M NaOH in a pH stat. *O*-Acyl groups were removed by treatment with 1 M NH_2OH at pH 10.0 for 1 h at room temperature. Excess reagents were separated from the modified protein by gel filtration on a Sephadex G-25 column equilibrated with phosphate-buffered saline.

(*B*) *Carbamylation.* Native CTX-1 was carbamylated according to the method of Stark (1972), at a potassium cyanate to protein molar ratio of 400:1 in phosphate-buffered saline adjusted to pH 8.0. After incubation for 48 h at room temperature (~23 °C), the modified protein was separated from the reagents by gel filtration as above.

(*C*) *Guanidination.* Native CTX-1 was guanidinated essentially by the method of Condrea et al. (1983). The cardiotoxin was treated with 0.5 M *O*-methylisourea at pH 10.8. The reaction was carried out at 4 °C for 72 h. Excess reagents were separated from modified CTX-1 by gel filtration on a Sephadex G-25 column as above.

Determination of Extent of Modification. After the modifications, the extent of modification of free amino groups was estimated by two methods. The Lys residues modified were measured as the loss of free amino groups. The unmodified amino groups were estimated by dansylation according to the method of Gray (1967); 1–2 nmol of native or modified polypeptides in 100 μL of 0.1 M sodium bicarbonate with 8 M urea was treated with 0.5 mL of dansyl chloride (20 mg/mL in acetone) for 3–4 h at 37 °C. Excess reagent was removed by gel filtration on Sephadex G-25 columns. The fluorescence emission was measured at 520 nm after excitation at 365 nm. The number of free amino groups was calculated from the fluorescence emission of dansylated products of the modified CTX-1, using the fluorescence emission of dansylated native CTX-1 as 100% for the free amino groups. The extent of modification by carbamylation and guanidination was also estimated by amino acid analyses, as homocitrulline and homoarginine, respectively.

Circular Dichroism Studies. To determine secondary structural changes caused by chemical modifications, CD studies for native and modified CTX-1 were performed with a Jasco J-500C spectropolarimeter. The cuvette chamber of the spectropolarimeter was continuously purged with pure nitrogen before and during experiments. Measurements were made in 0.1 M sodium phosphate buffer, pH 7.0, at room temperature. The secondary structural contents were estimated by a computer program based on the standard curve parameters described by Chang et al. (1978).

Binding Studies. Small unilamellar vesicles of PC or cardiolipin were prepared according to the method of Batenburg et al. (1985). Multilamellar vesicles obtained by hydrating the dry lipid films were sonicated with a Bronson B12 sonifier equipped with a 0.5-in. flat-top disruptor tip under nitrogen at 4 °C for 10 30-s intervals at 50 W. Unilamellar vesicles were isolated as the supernate after a 10-min centrifugation at 27000*g*.

The changes in intrinsic fluorescence of the single Trp residue (Trp-11) were recorded after binding of native or

[1] Abbreviations: CD, circular dichroism; CTX-1, cardiotoxin 1 isolated from *Naja nigricollis crawshawii* venom (Kini et al., 1987, 1988); PC, phosphatidylcholine.

Table I: Amino Acid Sequence of CTX-1 from *N. nigricollis crawshawii* Venom[a]

0	1	2	3
1	0	0	0
L K C N Q L I P P F W K T C P K G K N L C Y K M T M R A A P			
3	4	5	6
1	0	0	0
M V P V K R G C I D V C P K S S L L I K Y M C C N T D K C N			

[a] The one-letter notation of amino acids is used. The amino acid sequence is identical with that of the cardiotoxin previously isolated from *N. nigricollis* venom (Fryklund & Eaker, 1975).

modified CTX-1 to PC or cardiolipin unilamellar vesicles. After 3 min, the emission spectrum was measured with a Shimadzu RF-5000 recording spectrofluorophotometer, at the excitation wavelength of 280 nm. All fluorescence measurements were made at room temperature.

The energy transfer from Trp-11 of the polypeptide to anthracene incorporated in the membrane was determined according to the method of Uemura et al. (1983). Anthracene was added to the lipid solution in chloroform prior to the preparation of the dry film. The anthracene to phospholipid ratio in these vesicles was 1:80. Emission spectra were recorded 3 min after addition of the cardiotoxins.

Assay of Direct Lytic Activity. The direct lytic activity of the native and modified cardiotoxins was assayed as described earlier (Kini et al., 1988). Human erythrocytes were collected from citrated blood and washed with isotonic phosphate-buffered saline, pH 7.4. Twenty-microliter samples of proteins were incubated with 500 μL of resuspended erythrocytes (1:9 v/v in the same buffer) and 20 μM $CaCl_2$ in a total volume of 0.6 mL for 60 min at 37 °C. After incubation, 2 mL of the isotonic buffer was added, and the mixture was centrifuged at 4000*g* for 5 min. Liberated hemoglobin in the supernate was measured at 540 nm.

Release of Lactate Dehydrogenase from Platelets. Lysis of platelets was measured by release of lactate dehydrogenase as described earlier (Kini & Evans, 1988). Platelets were collected from normal human blood (with EDTA used as anticoagulant) by centrifugation and washed according to the method of Ardlie et al. (1971). A total of 400 μL of washed platelet suspension was incubated for 2 min with 20 μg of native or modified CTX-1 samples. This mixture was assayed directly for lactate dehydrogenase activity by the addition of 100 μL of 2.25 mM sodium pyruvate and 100 μL of 1.28 mM NADH (both dissolved in Tyrode's solution containing 1.8 mM Ca^{2+}). Enzyme activity was measured as the decrease in optical density at 340 nm.

Platelet Aggregation Studies. Inhibition of platelet aggregation was determined by electrical impedance measurements in a whole blood aggregometer (Chrono-Log Model 500) according to the method of Cardinal and Flower (1980). One milliliter of dilute citrated blood (1:1 dilution with phosphate-buffered saline) was incubated with 20 μL of protein sample at 37 °C for 2 min. Platelet aggregation was initiated with 1 μL of collagen (1 mg/mL).

Protein Estimation. Protein concentrations were estimated by amino acid analyses or by UV absorption at 280 nm. Native and modified cardiotoxins with a concentration of 1 mg/mL showed an absorbance of 1.60 at 280 nm.

Results

Amino Acid Sequence Determination. Cardiotoxins isolated from elapid snake venoms have 60 amino acid residues. All 60 residues were identified when 30 nmol of pyridylethylated CTX-1 was sequenced on the gas-phase sequencer with a repetitive yield of 94.0 ± 2.3% (data not shown). The sequence was identical with the previously sequenced *N. nigricollis* cardiotoxin (Table I) and shares high homology with the other 40 sequenced cardiotoxins (Dufton & Hider, 1983, 1988).

Table II: Summary of Modification of the Free Amino Groups of Cardiotoxin CTX-1

modification	charge on Lys side chain	no. of Lys modified		overall charge
		a	*b*	
none	positive			+9
succinylation	negative	9.0	ND[c]	−9
carbamylation	neutral	7.0	5.6	+3.4
guanidination	positive	9.0	8.8	+9

[a] Estimated by dansylation method. [b] Estimated by amino acid analyses. [c] ND, not determined (succinyllysine is hydrolyzed during amino acid analysis).

Table III: Effect of Modification of Amino Groups on the Secondary Structure of CTX-1

modification	structural content (%)[a]		
	α-helix	β-sheet	random coil
none	7.5	78	14.0
succinylation	11.0	79	10.0
carbamylation	9.0	81	10.0
guanidination	11.0	81	8.5

[a] The structural content was determined by CD according to the method of Chang et al. (1978).

Modification of Amino Groups. CTX-1 has nine Lys residues and two Arg residues. Since we propose that cationic residues are involved in the lytic activity of cardiotoxins, we modified the positively charged Lys residues to prepare nonlytic derivatives. Chemical modifications were performed to change the charge on the side chains of Lys residues: succinylation to obtain negatively charged succinyllysine residues; carbamylation to neutral homocitrulline residues; and guanidination to homoarginine residues with retention of the positive charges. The results of these modifications are summarized in Table II. Estimation of the free amino groups by dansylation agreed closely with the results of amino acid analyses. All the Lys residues were modified by succinylation and guanidination, whereas about six of nine residues were modified by carbamylation.

Effects of Modification on the Structure of CTX-1. Native cardiotoxin is a highly basic and hydrophobic polypeptide. The overall charge on the polypeptide is altered significantly by succinylation or carbamylation (Table II). After succinylation, the modified CTX-1 is a negatively charged polypeptide, and carbamylated CTX-1 is only slightly basic. However, guanidination retained the positive charges and thus the overall basicity of the cardiotoxin (Table II).

The secondary structures of the native and modified CTX-1 samples were examined by CD spectral analyses. The CD spectrum of native cardiotoxin shows an intense positive band at 192.5 nm and a negative trough near 225 nm with no positive band at 230 nm (Figure 1). This is similar to that of the identical cardiotoxin from *N. nigricollis* venom, and thus specifies it as a group I cardiotoxin (Grognet et al., 1988). No significant differences were observed between the CD spectra of the native and the Lys-modified cardiotoxins (Figure 1). Table III summarizes the secondary structural content calculated from the CD data. Only a small increase in the helical content and a small decrease in the random coil content were observed after the modifications. Thus, modification of the amino groups changed the charges but did not significantly affect the conformation of cardiotoxin CTX-1. This is not

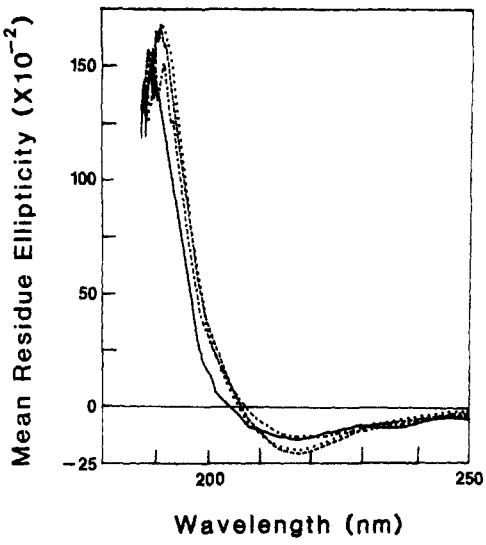

FIGURE 1: Circular dichroism spectra of native and modified cardiotoxin CTX-1 from *N. nigricolis crawshawii* venom. The spectra were recorded at 25 °C as described under Materials and Methods. Each spectrum is an average of four recordings. Toxin concentrations were in the range of 3–20 μM. A cell of 0.1-cm path length was used. (—) Native CTX-1; (---) succinylated CTX-1; (···) carbamylated CTX-1; (-·-) guanidinated CTX-1.

surprising, since one end of the molecule is stabilized by four disulfide bridges, whereas the other end is stabilized by hydrogen bonds between neighboring antiparallel β-strands. Since salt bridges do not have a role in the secondary structure of cardiotoxins (Rees et al., 1987; Steinmetz et al., 1988), the modification of charges should not alter the conformation of the molecule.

Effects of Modification on Binding of CTX-1 to Phospholipid Vesicles. The effects of modification of Lys side chains on binding of the cardiotoxin to cardiolipin and PC unilamellar vesicles were studied by fluorescence emission measurements. Intrinsic fluorescence measurements indicate the environment of Trp-11, and enhanced fluorescence reflects the transfer of parts of the peptide from a polar aqueous to an apolar intramembrane location. A marked increase in the emission intensity was observed when CTX-1 was bound to cardiolipin vesicles (Figure 2A), but no increase in the emission intensity, and hence no binding, was observed when CTX-1 was mixed with PC vesicles (Figure 2B). Similar results were observed with cardiotoxin II from *N. mossambica mossambica* venom (Batenburg et al., 1985). Succinylated CTX-1 bound preferentially to PC vesicles and not to cardiolipin vesicles (Figure 2C,D). Carbamylated cardiotoxin bound to both cardiolipin and PC vesicles (Figure 2E,F). Guanidinated CTX-1 resembles native cardiotoxin in binding preferentially to cardiolipin vesicles (Figure 2G,H).

To confirm the binding pattern of native and modified CTX-1, the effect of the intramembrane indole quencher anthracene was studied. The intrinsic fluorescence of the peptide was quenched by anthracene, and the fluorescence emission of anthracene was increased due to resonance energy transfer only when the peptide was bound to the vesicle (Figure 2). Thus, quenching and resonance energy transfer were observed when native CTX-1 or the carbamylated or guanidinated derivative were bound to cardiolipin vesicles. No increase in the emission of anthracene was seen when native or guanidinated CTX-1 was incubated with anthracene-incorporated PC vesicles (Figure 2). Thus, the studies of resonance energy transfer confirm the results of the binding studies.

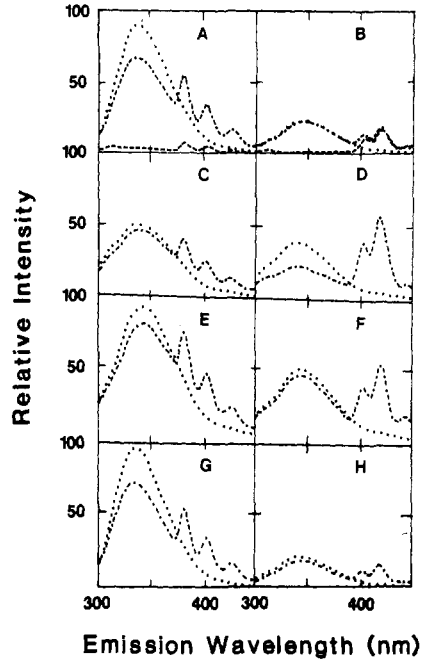

FIGURE 2: Emission spectra of native and modified CTX-1 after binding to phospholipid vesicles and resonance energy transfer to anthracene. Binding of native CTX-1 to cardiolipin (A) and PC (B) vesicles. Binding of succinylated CTX-1 to cardiolipin (C) and PC (D) vesicles. Binding of carbamylated CTX-1 to cardiolipin (E) and PC (F) vesicles. Binding of guanidinated CTX-1 to cardiolipin (G) and PC (H) vesicles. (---) Phospholipid vesicles with incorporated anthracene before the addition of polypeptides; phospholipid vesicles with (-·-) and without (···) incorporated anthracene after addition of the polypeptides. Concentrations: toxins, 3 μM; anthracene, 1.75 μM; phospholipid, 140 μM.

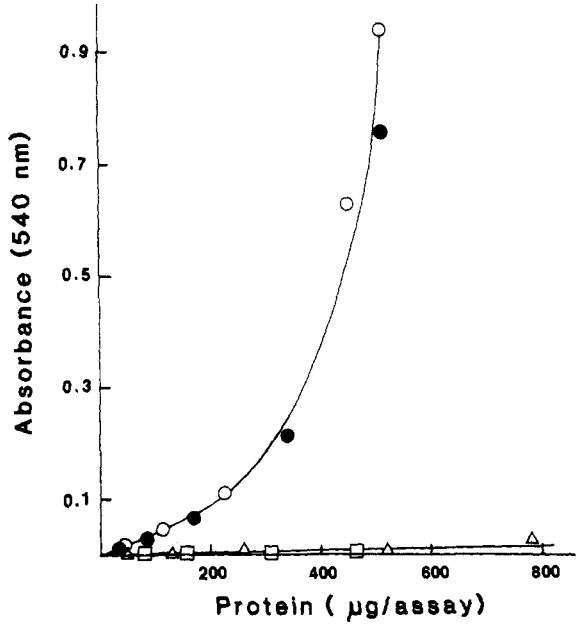

FIGURE 3: Direct lytic activity of native and modified CTX-1 from *N. nigricollis crawshawii* venom on human erythrocytes. (○) Native CTX-1; (□) succinylated CTX-1; (△) carbamylated CTX-1; (●) guanidinated CTX-1. Each point represents the average of triplicate analyses. Addition of distilled water for 100% lysis of erythrocytes gave an absorption of 3.0 at 540 nm.

Effects of Modification on the Lytic Activity of CTX-1. The effects of modification of Lys residues on the lytic activity of CTX-1 on human erythrocytes are shown in Figure 3. CTX-1 lost its cytotoxicity when the positively charged side chains of Lys residues were neutralized or changed to nega-

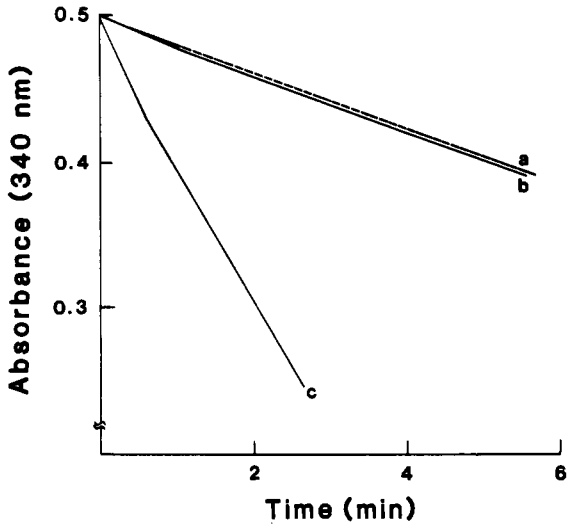

FIGURE 4: Release of lactate dehydrogenase from washed platelets by native and modified CTX-1 from *N. nigricollis crawshawii* venom. Twenty micrograms of each toxin was incubated with washed platelets, and released lactate dehydrogenase was measured as described under Materials and Methods. (a) Control (no toxin); (b) succinylated or carbamylated CTX-1; (c) native or guanidinated CTX-1. The tracings represent typical responses of at least four trials with each polypeptide.

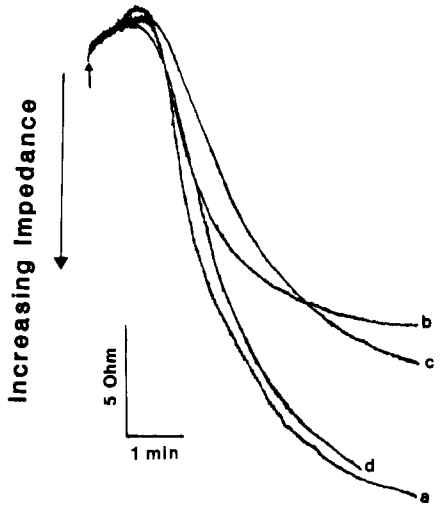

FIGURE 5: Potencies of inhibition of platelet aggregation by native and modified CTX-1. Final amount of each toxin in the reaction mixture was 20 μg. (a) Control (with no toxin); (b) native CTX-1; (c) guanidinated CTX-1; (d) succinylated or carbamylated CTX-1. The tracings represent typical aggregation responses of at least four trials with each polypeptide.

tively charged side chains. However, the guanidinated derivative retained complete lytic activity. We also determined the effect of CTX-1 and its derivatives on the lysis of platelets. Figure 4 shows that guanidinated CTX-1 retained the lytic activity of native CTX-1 while carbamylated and succinylated derivatives lost this activity.

Effects of Modification on the Antiplatelet Activity of CTX-1. We determined the effects of both lytic native and guanidinated CTX-1, and nonlytic carbamylated and succinylated CTX-1, on platelet aggregation induced by collagen (Figure 5). The nonlytic modified cardiotoxins lost their inhibitory activity while the guanidinated derivative retained the ability to inhibit platelet aggregation.

Discussion

Structure–Function Relationship of Cardiotoxins. Cardiotoxins and neurotoxins are two groups of polypeptides which are closely related in their structure, but show diversity in their

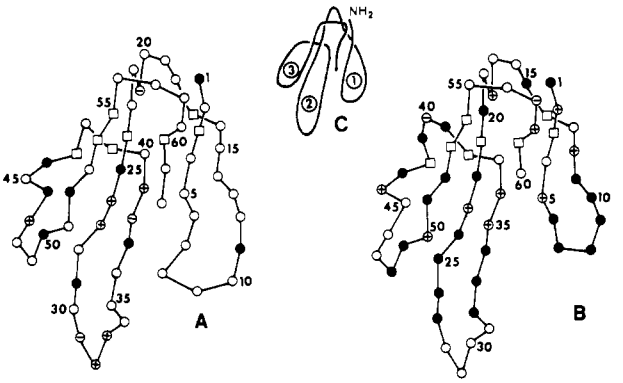

FIGURE 6: Distribution of hydrophobic and charged residues in short-chain neurotoxins and cardiotoxins. The backbone structures are extrapolations from the crystal structure of neurotoxin, representing neurotoxins (A) and cardiotoxins (B), respectively. The loop nomenclature is presented in (C). Rewritten from Dufton and Hider (1983) to show the distribution of hydrophobic and charged residues. (●) Hydrophobic residues [criteria for hydrophobicity described in Kini and Evans (1989)]; (⊕) positively and (⊖) negatively charged residues. The nature of each residue was determined from 45 sequences of neurotoxins and 50 sequences of cardiotoxins (Dufton & Hider, 1983, 1988) and occurs with at least 70% incidence in these polypeptides.

pharmacological effects. Amino acid sequences of about 80 neurotoxins (including both short- and long-chain types) and 50 cardiotoxins are known. Determinations of secondary structure have indicated the conformations of cardiotoxins are similar to those of neurotoxins (Dufton & Hider, 1977), especially short-chain neurotoxins. X-ray diffraction and proton NMR studies have also shown similarity in the three-dimensional structures of cardiotoxins and neurotoxins (Rees et al., 1987; Steinmetz et al., 1988; Labhart et al., 1988). The structural similarities make it difficult to understand the diversity in their pharmacological effects.

Some attempts have been made to resolve the structure–function relationships among neurotoxins and cardiotoxins, using the extensive amino acid sequence information. The common residues between these groups were thought to be responsible for the structural features while the residues present in one of the classes but not the other were considered critical in determining the specific pharmacological activity (Dufton & Hider, 1983). In a recent approach, individual amino acid substitutions and their effects on toxicity were considered for determining the structure–activity relationships of cardiotoxins (Dufton & Hider, 1988). This approach is complicated, and limited by the lack of suitable examples of cardiotoxins with the required substitutions. The approach therefore has only restricted applicability.

Dufourcq et al. (1982) and Bougis et al. (1983) predicted that loop 1 of cardiotoxin (Figure 6) is involved in binding to lipid vesicles. Synthetic amino-terminal peptides bind to phospholipid vesicles and compete with the cardiotoxins for binding. However, the loss of lytic activity after modification of Met-24 and Met-26 (Carlsson & Louw, 1978) cannot be explained by this prediction. This indicates the requirement of other parts of the molecule. The binding of amino-terminal peptides to phospholipid vesicles and their preference for negatively charged phospholipids (Dufourcq et al., 1982; Bougis et al., 1983) could be due to the hydrophobic and cationic nature, respectively, of the peptides. Binding, and hence the physical presence of the peptides on the phospholipid surface, can adequately explain the observed competition between the peptides and the cardiotoxins, but the structure–function relationships of cardiotoxins are still not clearly understood.

Prediction of the Cytolytic Region in Cardiotoxins. Earlier we identified a common cytolytic region, comprising a cationic site flanked by a hydrophobic site, in myotoxins, hemolysins, and antibacterial peptides (Kini & Iwanaga, 1986; Kini & Evans, 1989). The absence or unconserved substitution of residues in these sites in either natural or synthetic homologues caused the loss of cytolytic activity. Thus, we predicted that this region determines the cytolytic activity. However, attempts to extend the prediction to the cardiotoxins by direct comparison of amino acid sequences of lytic cardiotoxins and nonlytic neurotoxins failed to show any characteristic cytolytic region. We therefore considered the distribution of hydrophobic and charged residues in the three-dimensional structures. Figure 6 shows distinct differences between the structures of these closely related toxin groups. Strongly hydrophobic residues are located in the tip of loop 1, in the middle of loop 2, and along a portion of loop 3 of the cardiotoxins (Figure 6B). These residues appear to form a continuous hydrophobic surface which is available for interaction with the hydrophobic membrane surface. X-ray diffraction studies have shown that a continuous flat surface is formed by hydrophobic residues from loops 2 and 3 with side chains pointing in the same direction (Rees et al., 1987). There is a grouping of basic residues located in the middle of the molecule just above the hydrophobic surface. Only a portion of a cationic grouping, but no hydrophobic surface, is seen in the short neurotoxins (Figure 6A). The long-chain neurotoxins also lack the hydrophobic site (data not shown). The discrete absence of a hydrophobic site can explain the lack of cytolytic effects of the neurotoxins. Therefore, we predicted that the hydrophobic surface is involved in binding while the cationic site somehow determines the lytic activity of the cardiotoxins (Kini & Evans, 1989). In contrast to the other cytolysins, the amino acid residues forming the cytolytic region are not directly linked by peptide bonds and are grouped together only in the three-dimensional structure. Such a site is thus a "discontinuous" cytolytic region in contrast to the "continuous" regions of the other cytolysins.

Evidence for the Predicted Cytolytic Region. Our prediction is supported by the chemical modifications of amino acid residues of cardiotoxins. Oxidation of Met-24 and Met-26 results in the loss of both lytic activity and toxicity (Carlsson & Louw, 1978). These residues are located in the middle of the hydrophobic surface in loop 2 (Figure 6B). Their modification may reduce the binding ability of the derivative, resulting in loss of lytic activity. After nitration of either Tyr-22 or Tyr-51, the hemolytic potency remained unchanged (Carlsson & Louw, 1987; Gatineau et al., 1987), and modification of both these residues caused only a 2-fold loss in the lytic activity. These hydrophobic residues are also present in the neurotoxin (Figure 6A, residues 25 and 52, respectively) and thus seem to have no significant role in determining the lytic activity. Modification of Trp-11, located on the edge of the hydrophobic surface (Figure 6B), resulted in about 50% loss of lytic activity (Gatineau et al., 1987). Although Arg-36 was modified, the lytic activity of the derivative was not characterized (Carlsson, 1983). The results presented in this paper also support the prediction.

Effect of Modification of Cationic Residues on Binding to Phospholipids and Lytic Activity. In a previous paper, we predicted that the charges on the molecule probably contribute to the electrostatic interaction of the cytolysins, including cardiotoxins, with phospholipid membranes (Kini & Evans, 1989). The binding of the cardiotoxin derivatives to negatively charged cardiolipin and zwitterionic PC vesicles confirmed that the native CTX-1 and guanidinated CTX-1 bind preferentially to negatively charged vesicles and the succinylated derivative binds to PC vesicles, whereas neutral carbamylated CTX-1 binds to both lipid vesicles (Figure 2). These results support the contribution of the charges to the electrostatic interactions between the cardiotoxin and phospholipids. When the positive charges of the side chains of Lys residues were converted to neutral or negative charges, CTX-1 completely lost its lytic activity. However, when these positive charges were retained by guanidination, the modified CTX-1 showed the original lytic activity (Figures 3 and 4). In synthetic and natural homologues of cytolysins, in which the cationic residues were replaced by either a neutral or an anionic residue, the lytic activity was either partially or completely lost [discussed in detail in Kini and Evans (1989)]. However, when conservative changes in the amino acid residues were made, retaining both the hydrophobic and charge properties, the synthetic peptide showed strong lytic activity (Jaynes et al., 1988).

Since the derivatives retain the native conformation (Figure 1, Table III) and their ability to bind to phospholipid vesicles (Figure 2), the loss of lytic activity of the derivatives cannot be related either to the lack of a suitable conformation or to the inability to bind to vesicles. However, the loss of lytic activity directly correlates with the loss of the cationic site of the cardiotoxin, and thus shows the importance of the positive charges in determining cytolytic activity, although the specific Lys residues forming the cationic site remain to be identified.

Various mechanisms have been proposed to explain the cytolytic effects. Like other cytolysins [see Kini and Evans (1989)], cardiotoxins induce phase separation and have the ability to penetrate phospholipid vesicles (Faucon et al., 1983; Batenburg et al., 1985). A mismatch between the hydrophobic surface of the cytolysin and the lipid matrix might cause irregular lateral packing of hydrocarbon chains in phase segregation, resulting in increased osmotic fragility (Owicki & McConnell, 1979; Mouritsen & Bloom, 1984). Alternatively, the cytolysins might form transmembrane clusters in which the polar side chains are shielded from the hydrophobic environment (Vogel & Jahnig, 1986). Like other cytolysins, cardiotoxins are able to form "pores" (Ksenzhek et al., 1978). However, the exact mechanism of the lytic effect of cardiotoxins remains unclear.

Correlation between the Lytic Activity and Pharmacological Effects of Cardiotoxin. The cardiotoxins exhibit lethality, anticoagulant, and antiplatelet effects in addition to their lytic effects. No correlation was found between the toxicity and hemolytic activities of various cardiotoxins (Harvey, 1985). However, chemical modifications of Tyr-22 and/or Tyr-51, and Trp-11 have shown a positive correlation between the lytic activity and the lethality (Gatineau et al., 1987). A correlation coefficient of 0.95 could be calculated from the data. CTX-1 causes hemolysis at about 10^{-4} M concentration, but at 10^{-6} M, cardiotoxins cause depolarization of cardiac muscles. Therefore, hemolytic activity probably does not cause toxicity, although the role of "pore" formation in toxicity cannot be ruled out.

We proposed that the platelet effects of the cardiotoxins, i.e., inhibition of platelet aggregation observed in whole blood electronic aggregometers and initiation or potentiation of aggregation in turbidometric aggregometers, are due to their lytic activity (Kini & Evans, 1988). The nonlytic analogue neurotoxin II failed to inhibit platelet aggregation. As expected, the lytic polypeptides, native and guanidinated CTX-1, inhibited collagen-induced platelet aggregation whereas the nonlytic derivatives, succinylated and carbamylated CTX-1,

showed no effect on aggregation (Figure 5). These results confirm a direct correlation between the lytic activity and the platelet inhibitory effect of the cardiotoxin.

Conclusion. The structural analyses of lytic cardiotoxins and nonlytic neurotoxins indicate the presence of a specific cytolytic site in cardiotoxins. The cytolytic activity of cardiotoxins is determined by the presence of a cationic site flanked by a hydrophobic surface. The results presented in this paper confirm a significant role of the positive charges of Lys residues in determining the lytic activity of the cardiotoxins. The results also strongly reinforce the proposed lytic mechanism for antiplatelet effects.

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